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The TRAPP complex modulates the stress response by controlling stress granule composition and function

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The TRansport Protein Particle (TRAPP) complex is a multimolecular complex controlling membrane trafficking along the secretory, endocytic and autophagic pathways. With its multiple sites of action, TRAPP is in a strategic position to integrate the trafficking activity along these pathways in response to different cellular needs. By analyzing the response to different stress stimuli, we identified in TRAPP a key component of stress granules (SGs), membrane-less organelles that assemble in response to stress.

We provide different lines of evidence supporting a pivotal role for TRAPP in SG composition and function and in the integrated stress response. Firstly, the association of TRAPP with SGs orchestrates the adaptation to stress of the secretory pathway by inducing a slow-down of protein export from the ER (by trapping COPII on SGs) and the fragmentation of the Golgi complex (due to Rab1 inactivation, since TRAPP is a Rab1 GEF). Secondly, it increases the overall cell resistance to stress as it mediates the sequestering to SGs of pro-apoptotic factors (such as RACK1). Finally, the partitioning of TRAPP to SGs presents unique features, being under the control of cyclin-dependent kinase 1 and 2 and coupled with the cytosol-membrane cycle of TRAPP and COPII at ER exit sites. This renders the association of TRAPP with SGs, and thus the composition and function of the SGs themselves, finely tuned and conditional on the cell growth rate and the activity of the early secretory pathway.

Altogether, our findings reveal a new feature of SGs that is acquired through the recruitment of TRAPP: a high grade of plasticity that tailors their composition and function to different cellular metabolic, growth or activity states.

Introduction

The TRAPP (transport protein particle) complex is a conserved multimolecular complex intervening in multiple segments of membrane trafficking along the secretory, the endocytic and the autophagy pathways (Kim et al., 2016).

Originally identified in yeast as a tethering factor acting in ER-to-Golgi trafficking, it was subsequently discovered to act as a GEF for Ypt1 and Ypt31/32 in yeast and for Rab1 and possibly Rab11 in mammals (Westlake et al., 2011; Yamasaki et al., 2009; Zou et al., 2012). The TRAPP complex has a modular composition, and is present as two forms in mammals: TRAPPII and TRAPPIII which share a common heptameric core (TRAPPC1, TRAPPC2, TRAPPC2L, TRAPPC3, TRAPPC4, TRAPPC5, TRAPPC6) and additional subunits specific for TRAPPII (TRAPPC9/ TRAPPC10) or for TRAPPIII (TRAPPC8, TRAPPC11, TRAPPC12, TRAPPC13) (Sacher et al., 2018). TRAPPII has been implicated in late Golgi trafficking while TRAPPIII has a conserved role in the early secretory pathway (ER-to-Golgi) and in autophagy Yamasaki (Scrivens et al., 2011; Yamasaki et al., 2009). Recent lines of evidence have expanded the range of activities of the TRAPP complex by showing that it takes part in a cell survival response triggered by agents that disrupt the Golgi complex (Ramírez-Peinado et al., 2017)and can drive the assembly of lipid droplets in response to lipid load (Li et al., 2017).

The importance of the TRAPP complex in humans is testified by the deleterious consequences caused by mutations in genes encoding distinct TRAPP subunits. Mutations in TRAPPC2L, TRAPPC6A, TRAPPC6B, TRAPPC9 and TRAPPC12 cause neurodevelopmental disorders leading to intellectual disability and dysmorphic syndromes (Harripaul et al., 2018; Khattak and Mir, 2014; Milev et al., 2018, 2017, p. 12; Mohamoud et al., 2018), mutations in TRAPPC11 lead to ataxia (Koehler et al., 2017), and mutations in TRAPPC2 lead to the spondyloepiphyseal dysplasia tarda (SEDT) (Gedeon et al., 1999).

SEDT is characterized by short stature, platyspondyly, barrel chest and premature osteoarthritis that manifest in late childhood/prepubertal age. We have shown that pathogenic mutations or deletion of TRAPPC2 alter the ER export of procollagen (both type I and type II) and that TRAPPC2 interacts with the procollagen escort protein TANGO1 and regulates the cycle of the GTPase Sar1 at the ER exit sites (ERES; (Venditti et al., 2012)). The Sar1 cycle in turn drives the cycle of the COPII coat complex, which mediates the formation of carriers containing neo-synthesized cargo to be transported to the Golgi complex. While this role of TRAPPC2 in the ER export of PC might explain the altered ECM

deposition observed in patients cartilage (Venditti et al., 2012; Tiller et al., 2001), it leaves unexplained the late onset of the disease signs as well as the development of precocious osteoarthritis. We hypothesized that the latter could be due to an inability to maintain long-term cartilage tissue homeostasis, possibly due to an impaired capacity of chondrocytes to face the physiological stresses that underlie and guide their development and growth. These include mechanical stress that can induce oxidative stress leading to apoptosis (Henrotin et al., 2003; Zuscik et al., 2008).

Indeed, the TRAPP complex, with its multiple sites of action along the secretory and autophagic pathways and its multiple molecular partners (including COPII and COPI), occupies a strategic position to integrate the activities along these pathways in response to different stimuli including physiological and pathological stressors. Here, by analyzing the cell response to different stresses, we show that the TRAPP complex is a component of stress granules (SGs), membrane-less organelles that assemble in response to stress (Protter and Parker, 2016). The recruitment of TRAPP to SGs has multiple impacts on the stress response as it induces the sequestration of Sec23/Sec24 (the inner layer of the COPII complex) onto SGs thus inhibiting trafficking along the early secretory pathway, leads to the inactivation of the small GTPase Rab1 with a consequent disorganization of the Golgi complex, and is required for the recruitment of signalling proteins, such as RACK1, to SGs thus contributing to the anti-apoptotic role of SGs. Interestingly, TRAPP and COPII recruitment to SGs only occurs in actively proliferating cells and is under control of cyclin-dependent-kinases (CDK 1 and 2). We identified in Sec31 one of the Cdk targets that is able to modulate the COPII cycle at the ERES. CDK inhibition stabilizes TRAPP and COPII at the ERES and reduces their cytosolic pool, i.e, the one that is sequestered onto SGs.

In conclusion, we highlight a hitherto undisclosed property of SGs: a differential and modular composition dependent on cell growth activity that reflects the ability of cells to commensurate their response to stress to their proliferation state and energy demands., and we have identified in the TRAPP complex a key element in conferring this adaptive plasticity to SG.

Results

TRAPP redistributes to SGs in response to different stress stimuli

TRAPP components such as TRAPPC2 and TRAPPC3 have been shown to be associated with ERES under steady state conditions (Venditti et al., 2012). We found that exposure of chondrocytes (Figure 1A) or HeLa cells (Figure 1B) to an oxidative stress such as treatment with sodium arsenite (SA) led to dissociation of TRAPPC2 from ERES and a strong relocalization to roundish structures. Since oxidative stress is known to lead to the formation of stress granules (SGs; Anderson and Kedersha, 2002), we considered the possibility that these TRAPPC2-positive structures might be SGs. Indeed, co-labeling with an anti-eIF3 antibody, a canonical SG marker (Aulas et al., 2017), showed the co-localization of TRAPPC2 with eIF3 after SA treatment (Figure 1A,B). Other TRAPP components, such as TRAPPC1 and TRAPPC3, exhibited the same response to SA treatment (Figure 1C). TRAPP recruitment to SGs occurs also in response to heat shock (Figure 1—figure supplement 1A), and in different cell lines (Figure 1—figure supplement 1B,C). This appears to be a conserved process since it also occurs in yeast cells exposed to heat stress (Figure 1—figure supplement 2, Figure 1—supplement video 1 and 2). Of note, the association of TRAPP with SGs is fully reversible after stress removal (Figure 1—figure supplement 3).

We followed the dynamics of TRAPPC2 redistribution to SGs in response to stress. The appearance of eIF3-positive SGs occurred 7 min after SA treatment (Figure 1D). TRAPPC2 re-localization to SG began 15 min after the exposure to stress and gradually increased over time, with massive recruitment occurring 60 min after treatment, and thus lagged behind the initial assembly of SGs (Figure 1D).

We analyzed the TRAPP complex by gel filtration and found that the oxidative stress did not affect the overall integrity of the complex. However, TRAPP components were also found in higher molecular weight fractions in stressed cells (Figure 1E) possibly reflecting the SG-associated TRAPP since the same fractions also contained the SG component TIAR-1 (Figure 1E). Together, these results led us to conclude that following stress the TRAPP complex becomes a SG component.

TRAPPC2 is required for recruiting COPII to SGs

The translocation of TRAPP to SGs prompted us to investigate whether other components of membrane trafficking machineries behaved similarly. We screened different coat complex components (COPI, COPII, clathrin adaptors, clathrin), and other cytosolic proteins associated with the exocytic and endocytic pathways. Out of the 15 proteins tested, only components of the inner layer of the COPII coat, Sec24 (Figure 2A, B) and Sec23 (Figure 2—figure supplement 1A), but none of the others (Figure 2C, and Figure 2—figure supplement 1B-N, Figure 2—figure supplement 2) associated with SGs. Notably, Sec24 was recruited to SGs with kinetics similar to those of the TRAPP complex (Figure 2A).

Since TRAPP and COPII are known to interact and TRAPP is recruited to ERES in a Sar1- and COPII-dependent manner (Lord et al., 2011; Venditti et al., 2012), we asked whether COPII and TRAPP recruitment to SGs is interdependent. The depletion of the COPII inner layer proteins Sec23 and Sec24 had no impact on the recruitment of the TRAPP complex to SGs (Figure 2D,E) while the depletion of the entire TRAPP complex (by KD of TRAPPC3, which destabilizes the entire complex,) or of the TRAPPC2 subunit (by KD of TRAPPC2 that leaves unaltered the rest of the TRAPP complex, (Venditti et al., 2012)) abrogated the recruitment of COPII components to SGs (Figure 2F). We also noted that TRAPP-KD resulted in smaller SGs, a phenomenon that is analyzed in more detail below. These results indicated that the TRAPP complex, through its component TRAPPC2, drives the recruitment of COPII to SGs.

COPII components have been reported to associate with membrane-less organelles called “Sec bodies” in Drosophila S2 cells (Zacharogianni et al., 2014) . Sec bodies, however, form in response to prolonged starvation (but not to oxidative stress) and are distinct from SGs since they do not contain ribonucleoproteins (RNPs). The recruitment of COPII to Sec bodies in Drosophila depends on Sec16, another component of ERES. However, we found that in mammalian cells Sec16 neither significantly associates with SGs (Figure 2—figure supplement 2A,B) nor is it required for the recruitment of COPII to SGs (Figure 2—figure supplement 2C).

COPII proteins exhibit dynamic association/dissociation with ERES membranes (D’Arcangelo et al., 2013). An indirect assessment of the membrane-cytosol cycle of the endogenous COPII components may be obtained by evaluating their leakage

from permeabilized cells (Kapetanovich et al., 2005), as the extent of leakage is proportional to the rate of membrane dissociation. Under steady state conditions, the majority of COPII components is lost from cells 6 min after permeabilization (Figure 3A). By contrast, COPII remained associated with SGs in permeabilized SA-treated cells (Figure 3A), indicating that the SG-associated pool of COPII is much less dynamic than the ERES-associated pool.

We then assessed the relationship between the pool of COPII/TRAPP cycling at the ERES and that recruited to SGs. To this end we stabilized the ERES association of COPII and TRAPP by expressing either a constitutively active GTP-bound Sar1 mutant (Sar1-H79G) or decreasing the rate of GTP hydrolysis on Sar1 by depleting Sec31, which is a co-GAP that potentiates by an order of magnitude the Sar1 GAP activity of Sec23-24 (Bi et al., 2007). Under both conditions COPII and TRAPP were more tightly associated with ERES, while their translocation to SGs upon exposure to stress was significantly reduced (Figure 3B,C). Thus, the extent of COPII/TRAPP recruitment to SG depends on the rate of its cycling at the ERES.

The association of TRAPP is under the control of CDK1/2

To investigate the mechanisms that could mediate the recruitment of TRAPP to SGs, we performed a proteomics analysis of the TRAPPC2 interactors (see Materials and methods). This analysis (Figure 4A, Table), while confirming the known interactors of TRAPPC2 (e.g. TRAPP complex components, Rab1, COPII and COPI), revealed the presence of many RNA binding proteins (RBPs), including those with a central role in the assembly of SGs (Jain et al., 2016). Both known interactors and some RBPs were confirmed by Western blot analysis (Figure 4—figure supplement 1). This suggested that, as described for other components of SGs, TRAPPC2 may be recruited to growing SGs by a piggyback mechanism (Anderson and Kedersha, 2008), i.e. via the interaction with RBPs that are components of SGs.

We next looked for the mechanisms regulating this recruitment. To dissect the regulation of TRAPP/COPII recruitment to SGs, we screened a library of kinase inhibitors for their ability to affect the re-localization of COPII to SGs. HeLa cells were pre-treated with kinase inhibitors for 150 min and then exposed to SA for a further 30 min in the continuous presence of the inhibitors. Out of the 273

inhibitors tested, 194 had no effect on SG formation or on COPII recruitment, 13 were generally toxic (65% mortality) at the tested concentrations, one (AZD4547) affected the formation of SGs, while the remaining 32 specifically inhibited (by at least 35%) the association of Sec24C with SGs without affecting SG formation (Figure 4B). Enrichment analysis on this last group of inhibitors highlighted that CDK inhibitors were the most represented class (Figure 4C). Western blot analysis confirmed that CDK activity was impaired in cells treated with the CDK inhibitors (Figure 4D). In addition, we observed that there is an increase in CDK activity in SA-treated cells that was sensitive to CDK inhibitors (Figure 4D).

The CDK protein kinase family is composed of 21 members (Malumbres, 2014) but the available inhibitors have limited selectivity (Asghar et al., 2015). To identify which specific CDK might be involved in Sec24C recruitment, we compared the potency of the CDK inhibitors (Flavopiridol hydrochloride, SNS-032, Dinaciclib, AT7519, PHA-793887, ADZ5438, JNJ-7706621, PHA-767491, BMS-265246, PHA-848125, Roscovitine, Palbociclib, BS-181HCl) in the Sec24C recruitment assay with their ability to inhibit the different CDK isoforms, as described by Selleckchem (https://www.selleckchem.com/CDK.html). This analysis revealed that CDK1 and 2 were the ones commonly targeted by the inhibitors that most potently affected Sec24C recruitment to SGs (Figure 5A), thus suggesting that CDK1/2 are the most likely CDKs controlling COPII recruitment to SGs. This was confirmed by the observation that down-regulating the expression of CDK1 and CDK2 by siRNA inhibited COPII recruitment to SGs (Figure 5B). The three best hits (Flavopiridol, Dinaciclib, SNS032, Figure 5A,C) were assayed using a dose-response analysis and were found to be effective at concentrations as low as 100 nM (Figure 5D), thus speaking in favor of a specific effect on CDK1/2. As a negative control, the specific CDK7 inhibitor (BS-181HCl) was ineffective, even at the highest concentration tested (Figure 5D).

Since COPII recruitment to SGs is driven by TRAPP recruitment (Figure 2F), we hypothesized that CDK1/2 inhibitors would also inhibit TRAPP recruitment. In line with our expectations, TRAPP re-localization to SGs was reduced in cells treated with CDK1/2 inhibitors (Figure 5E).

Altogether, these results indicate that a signaling pathway mediated by CDK1/2 controls the recruitment of TRAPP and COPII to SGs.

The recruitment of TRAPP and COPII to SGs occurs in actively proliferating cells

The observation that the recruitment of TRAPP and COPII to SGs is under control of CDK1/2 suggested that it could be linked to the cell proliferation. We explored this possibility by decreasing the cell proliferation rate in different and independent ways: by reducing nutrient availability, by increasing cell confluence, or by inducing a switch from a proliferation to a differentiation state.

First, cells were subjected to prolonged nutrient starvation (HBSS for 8 hr) and then were exposed to SA for 30 min. Under these conditions, which inhibited cell proliferation, TRAPP/COPII association with SGs was reduced, while SGs formed regularly (Figure 6A,B).

Second, HeLa cells were seeded at different confluency and then treated with AS for 30 min. Strikingly, the extent of confluency was inversely related to the extent of TRAPP/COPII association with SGs and to the degree of proliferation (Figure 6C).

Third, we monitored the recruitment of TRAPP/COPII to SGs in podocyte cells expressing a temperature sensitive LTA-SV40 that actively proliferate at the permissive temperature of 33ºC but arrest proliferation and differentiate at the restrictive temperature of 37ºC (Saleem et al., 2002) (Figure 6D, Figure 6—figure supplement 1). While TRAPP and COPII associate with SGs in undifferentiated podocytes in response to stress, this association was reduced in differentiated podocytes (Figure 6E). Of note, the proliferation to differentiation switch, as described (Saurus et al., 2016), is also accompanied by a decline in CDK activity (Figure 6F). These data indicate that the association of COPII and TRAPP with SGs occurs only in proliferating cells and requires active CDK1/2 signaling.

CDK1 and 2 control TRAPP/COPII recruitment to SGs by modulating their cycle at the ERES.

We next investigated the nature and the possible target(s) of the pathway involving CDK1/2 that controls the recruitment of TRAPP/COPII complexes to SGs. As shown above (Figure 3), the recruitment of TRAPP/COPII to SGs is conditioned by the dynamics of the COPII cycle at ERES. We hypothesized that CDK inhibition

might prevent the recruitment of TRAPP/COPII to SGs by stabilizing their association with the ERES. Indeed, we found that the pool of TRAPP and COPII associated with ERES is higher, while the cytosolic pool of the two complexes is reduced, in cells treated with the CDK1/2 inhibitors as compared to untreated cells (Figure 7A). The analysis of the pool associated at ERES by a permeabilization assay confirmed that the CDK1/2 inhibitors strongly increased the amount of COPII associated with ER membranes-(Figure 7B,C).

To understand how CDK1/2 might affect the COPII cycle at the ERES, which in turn is dependent on the cycling of the small GTPase Sar1, we focused on Sec31 as a possible target for a number of considerations. First, Sec31 is required for proper Sar1 cycling at the ERES (Bi et al., 2007); second we have shown that Sec31 is required for the translocation of COPII/TRAPP to SG (Figure 3C).; finally, Sec31 is a target of CDKs in yeast (Holt et al., 2009) and in *Trypanosoma brucei* where it has been shown that Sec31 phosphorylation by CRK1 (the CDK ortholog in *T. brucei*) is required for anterograde trafficking (Hu et al., 2016). In mammals, Sec31 activity is modulated by post-translational modifications including phosphorylation on multiple sites (Koreishi et al., 2013) one of which (serine 799) is included in a consensus motif for CDK phosphorylation. We tested whether Sec31 could be a substrate for CDK using an antibody specific for phosphoSer CDK substrates. We found that Sec31 is indeed a target for CDKs (Figure 7D), and that its CDK-dependent phosphorylation is increased upon SA treatment and decreased by the CDK inhibitor Dinaciclib (Fig. 7D). A further correlation between the phosphorylation of Sec31 and the recruitment of COPII and TRAPP to SGs is provided by the observation that the extent of CDK-dependent phosphorylation is dampened in differentiated as compared with proliferating podocytes (Figure 7E). Altogether, the above results suggest that phosphorylation of Sec31 by CDKs might modulate COPII membrane association thus regulating the availability of the cytosolic pool able to phase-separate to SGs.

The recruitment of TRAPP and COPII to SGs slows down ER export and induces the fragmentation of the Golgi complex

We investigated the impact of the recruitment to TRAPP/COPII to SGs on the secretory pathway.

We first analysed the ER export of procollagen type I (PC-I), which is known to be regulated by COPII and TRAPP (Gorur et al., 2017; Venditti et al., 2012). PC-I transport can be synchronized by temperature, being unfolded and blocked in the ER at 40°C while properly folded and assembled and able to be exported from the ER, transported to the Golgi complex (GC) and secreted at 32°C ((Mironov et al., 2001) and Methods). Note that incubation at 40°C does not cause SG aggregation. Human fibroblasts were incubated at 40°C, left untreated or treated with SA, which induces the recruitment of Sec24C to SGs (Figure 8A). PC-I is blocked in the ER under both conditions (Figure 8A). Monitoring of PC-I transport after the shift to 32°C showed that while PCI reaches the GC in untreated cells, it remains blocked in the ER in SA-treated cells (Figure 8A).

To investigate to what extent the inhibition of cargo export from the ER induced by oxidative stress is dependent on COPII/TRAPP sequestering onto SGs, we developed an assay based on the use of an engineered form of a reporter cargo (Chen et al., 2013) whose exit from the ER is inducible by UV light. We compared the ER export of VSVG in control cells and in SA-treated cells either under conditions that induce a strong recruitment of COPII/TRAPP to SGs (SA treatment for 30 min) or under conditions that induce SG formation but attenuate this recruitment (SA treatment for 10 min). After UV light induction, VSV-G exited the ER and reached the Golgi complex to comparable extent in control cells and in cells treated with SA under conditions that attenuate the recruitment of COPII to SGs, while the ER export of VSVG in response to UV light was severely retarded in cells treated with SA under conditions where COPII was strongly recruited to SGs (Figure 8B, D).

We then tested whether COPII and TRAPP maintain their functionality once released from SGs upon stress removal, which can be dissolved after washout of SA, as mentioned above (Figure 1—figure supplement 3). Cells were treated with SA for 30 min, the SA was washed out, and cells were left to recover for 180 min in the presence of cycloheximide to prevent *de novo* synthesis of TRAPP and COPII components. Under these conditions SGs were resolved, COPII returned to its

native location (ERES/cytosol) and cells completely recovered their capability to transport cargo to the Golgi apparatus (Figure 8C,D). These data indicate that sequestration of COPII/TRAPP onto SGs halts ER-to-Golgi trafficking while removal of the stress releases COPII/TRAPP and allows trafficking to resume.

COPII and TRAPP not only control ER export but are also needed to maintain the organization of the GC. In particular, the TRAPP complex acts as GEF for Rab1, a GTPase with a key role in the organization and function of the GC (Tisdale et al., 1992; Wilson et al., 1994). We monitored Golgi complex morphology in response to SA by following the distribution of the early Golgi marker GM130. Strikingly, the GC starts to fragment after 30 min and completely redistributes throughout the cytoplasm after 60 min of SA treatment (Figure 9A). This time window overlaps with the progressive recruitment of the TRAPP complex onto SGs (Figure 1D). To assess whether the Golgi fragmentation induced by oxidative stress was due to sequestration of TRAPP/COPII onto SGs, we analyzed the morphology of the GC in cells exposed to oxidative stress under conditions that prevent COPII/TRAPP recruitment to SGs, i.e. treatment with CDK inhibitors or highly confluent cells. We found that CDK inhibitors prevented the fragmentation of the GC in cells exposed to oxidative stress (Figure 9B), and that oxidative stress had no impact on the organization of the Golgi complex in highly confluent cells (Figure 8C). These results indicated a causal link between TRAPP/COPII sequestration to SGs and Golgi fragmentation.

To gain insight into the nature of the fragmentation of the Golgi complex, we performed ultrastructural analysis that revealed the presence of residual swollen cisternae with the loss of stacked structures and extensive vesiculation in SA-treated cells (Figure 9D), a situation that is reminiscent of the effect of Rab1 inactivation (Wilson et al., 1994). We hypothesized that the dismantling of the Golgi complex could have been due to impaired Rab1 activation as a consequence of TRAPP (the Rab1 GEF) sequestering to SGs. We monitored Rab1 activity by using an antibody that specifically recognizes the GTP-bound Rab1 and found, indeed, that the fraction of active Rab1 (i.e. the GTP-bound form) is progressively reduced in cells exposed to SA (Figure 9E). Overexpression of wt Rab1 reduced fragmentation/vesiculation of the GC induced by oxidative stress without

affecting the capacity of TRAPP to migrate to SGs (Figure 9F, Figure 9—figure supplement 1), establishing a causal a link between Rab1 inactivation and Golgi fragmentation.

The TRAPP complex imposes SG size, composition, and function

We finally investigated whether TRAPP is an inert component of SGs or whether it plays any role in their assembly/function. Indeed, as noted above (Figure 2F), TRAPP-KD resulted in smaller and more numerous SGs, suggesting that the TRAPP complex could exert a role in the overall composition and function of SGs. Depleting the entire TRAPP complex or only TRAPPC2 (through siRNA-mediated depletion) decreased the size of SGs (Figure 2F and Figure 10A). Microinjection of a TRAPPC3-blocking Ab (Yu et al., 2006; Venditti et al., 2012) had a similar effect (Figure 10B). Under these conditions the size of the SGs recall that of "immature SGs" (Wheeler et al., 2016).

In fact the processes that lead to SG nucleation, i.e. impaired protein synthesis or phosphorylation of eIF2alpha induced by oxidative stress, were not affected by TRAPP depletion (Figure 10—figure supplement 1A,B). Instead, the successive increase in size of the nucleated SGs (i.e. SG maturation) was impaired in TRAPP depleted cells, consistent with our observation that TRAPP is recruited after the initial formation of SGs and occupies an outer position in the SGs (figure 10C). A feature of SG maturation is the acquisition of an array of signaling molecules (which can be either hyperactivated or deactivated) to drive the pro-survival program. This is the case of the energy sensor mTORC1 (through the sequestration of its partner Raptor) and the kinase RACK1 (Arimoto et al., 2008; Thedieck et al., 2013). To evaluate whether the absence of TRAPP could affect SG maturation and/or signaling function by interfering with the recruitment of these proteins, we checked the localization of Raptor (Figure 10D) and RACK1 (Figure 10E, Figure 10—figure supplement 2) in TRAPP- and or TRAPPC2-depleted cells subjected to oxidative stress. We observed that the amount of these signaling proteins sequestered to SGs was strongly reduced by TRAPP depletion, thus indicating that the TRAPP complex is necessary to generate "mature" SGs that can accomplish a fully functional stress response. Localization of RACK1 and Raptor was also evaluated in a context of CDK inhibition. Again, treatment with CDK

inhibitors strongly reduced Raptor (Figure 10F) and RACK1 (Figure 10G) sequestration, further supporting that the TRAPP complex is needed for SG maturation.

SGs are part of a pro-survival program that aims to save energy to cope with stress insults. Interfering with this program by impairing SG formation sensitizes cells to apoptosis (Takahashi et al., 2013). We thus assayed stress resistance in cells with an impaired capability to recruit TRAPP to SGs, i.e. upon CDK inhibition. As shown in Figure 10H, CDK inhibition significantly sensitized cells to cell death, indicating that the presence of TRAPP at SGs is required for their anti-apoptotic role and highlighting the TRAPP complex as a novel player in the cell response to stress.

Discussion

We have described a branch of the integrated stress response that is dependent on the assembly of SGs and leads to a slow-down of ER export and disorganization of the Golgi complex. A key component of this branch is the TRAPP complex, a multimolecular complex that intervenes in multiple membrane trafficking steps.

We have shown that TRAPP is massively recruited to SGs in response to different acute stress stimuli and in turn recruits the inner layer of the COPII complex, the protein complex that drives the export of newly synthesized proteins from the ER, to SGs. The mechanism underlying the recruitment of TRAPP/COPII is likely to be a piggyback mechanism linked to the ability of TRAPPC2 to interact with multiple RNA binding proteins, a mechanism that has been described for many other components of SGs (Anderson and Kedersha, 2008).

The functional consequences of the recruitment of TRAPP/COPII to SGs are double-edged since on the one hand they impact on the function and organization of the secretory pathway while on the other they impact on the composition and function of the SGs. The recruitment/sequestering of two complexes, one with a driving role in ER export and another acting as a GEF for Rab1, a master GTPase orchestrating the organization of the Golgi complex, induces, not surprisingly, both a slow-down of the ER-to-Golgi transport of newly synthesized proteins and

the disorganization of the structure of the Golgi complex. Importantly, both of these changes are completely reversible since ER export resumes and proper Golgi morphology is restored upon stress removal and relocation of the two complexes to their natural sites. While the sequestering of TRAPP/COPII impinges mainly on ER export and on the early secretory pathway, the recently described delocalization of the TGN-located PARP12 to SGs affects mainly late Golgi compartments (Catara et al., 2017).

The consequence of sequestering of TRAPP/COPII to SGs, i.e., an immediate halt in secretory activity, an energetically costly process, can be seen as a way to limit energy expenditure upon acute stress but also to prevent the ER export of newly synthesized proteins that might have been damaged/misfolded by the acute stress before an adequate UPR is installed and becomes operative.

An unanticipated feature of the stress response we have reported here is its dependence on the proliferation status of the cells. Indeed, the sequestration of TRAPP and COPII to SGs occurs only when the acute stress impinges on actively proliferating cells. Cells with a slow/halted proliferation rate still form SGs in response to acute stress stimuli, but do not delocalize TRAPP/COPII from their steady state sites, i.e., the ERES, to SGs. We have used multiple strategies to slow down (or halt) proliferation: nutrient deprivation, a high cell density, or a switch from a proliferation to a differentiation program. Under all circumstances we observed a very tight correlation between the extent of recruitment of TRAPP/COPII to SGs and the proliferation rate, indicating that acquisition of components of the secretory machinery does not occur by default as a consequence of the assembly of the SGs but is an active process that is subjected to specific regulation. We found that this regulation involves the activity of CDK1&2, since CDK1&2 inhibitors and/or CDK1&2 downregulation prevented the relocation of TRAPP/COPII to SGs. With regard to relevant mechanisms controlled by CDKs, these may be multiple and co-operative. Some components of SGs are CDKs targets, including some of the TRAPPC2 interactors (Moujalled et al., 2015) and the CDK-dependent phosphorylation of these targets has been shown to impinge on the composition of the SGs. Additionally, the function of the ERES and

some of the components of the COPII coat are controlled by CDKs. A member of the CDK family, PCTAIRE, is recruited to ERES via its interaction with Sec23 and controls ER export (Palmer et al., 2005) and Sec31, a component and a regulator of COPII cycle, is a substrate for CDKs in yeast and in Trypanosoma, and, as we have shown here, also in mammalian cells (Figure 7C,D). Further, the phosphorylation of Sec31 in response to growth factor and mitogenic signals has also been reported (Dephoure et al. 2008; Olsen et al., 2006).

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We have shown here that, consistent with the described role of Sec31 as a co-GAP for Sar1, Sec31 function is required to control the COPII cycle at the ERES, since Sec31 depletion induces a tighter association of the inner COPII components Sec23-24 with ERES and prevents their translocation to SGs. In fact, our results indicate that the extent of translocation of COPII/TRAPP to SGs is directly proportional to their cycling rate at the ERES membranes. We have shown that this rate is diminished by CDK inhibitors, but also, importantly, in starved cells as compared to actively proliferating cells. The regulation of the cycling rate of ERES components by growth factors and growth factor-dependent signaling has been reported in large scale and focused studies (Farhan et al., 2010; Tillmann et al., 2015). Thus, the activity of the ERES, as measured by their number and by the rate of cycling of ERES components, is stimulated under conditions that require maximal efficiency of output from the ER, such as during cell size increase and organelle expansion. If cells are affected by an acute stress under these conditions, then the fast cycling COPII components in actively growing cells are temporarily sequestered to the SGs to instantly slow down ER output and reduce energy consumption. By contrast, we found that nutrient starvation imposes a slow-down of the COPII/TRAPP cycle and, by doing so, prevents COPII/TRAPP translocation to SGs. Indeed, a profound remodeling of ERES during starvation has been reported where COPII and TRAPP are diverted from their role in the secretory pathway to their role in autophagosome biogenesis (Ge et al., 2017; Imai et al., 2016; Kim et al., 2016; Lamb et al., 2016; Ramírez-Peinado et al., 2017; van Leeuwen et al., 2018). We hypothesize that the rate of cycling of COPII at its site of action in autophagosome biogenesis is lower than its rate at the “conventional” ERES under feeding conditions and this in turn may prevent its sequestration to

SGs upon stress exposure. The lack of sequestration of TRAPP/COPII in starved cells exposed to stress would allow COPII and TRAPP to function and contribute to the autophagy process, a process that leads to nutrient recycling and energy production, desirable events in cells exposed to stress.

Finally, we have shown that TRAPP is not a "passive" constituent of SGs since its recruitment to SGs is required for the proper maturation of these membrane-less organelles. Preventing the recruitment of TRAPP to SGs or depleting TRAPP does not impair the formation of SGs *per se* but hampers their maturation, as evaluated by their size (smaller SGs in the absence of TRAPP) and composition. In particular, key signaling components, such as RACK1 and Raptor, are no longer recruited to SGs in TRAPP-depleted cells. The consequences of impaired SG maturation in TRAPP-depleted cells consist in lower resistance to stress and a higher tendency to undergo apoptosis. This role of TRAPP in conferring higher resistance to stress, especially in highly proliferating cells, could be extremely relevant in proliferating chondrocytes, which are key components of the epiphyseal growth plate and whose malfunction, as a consequence of ill-tolerated repetitive stresses, could lead to the progressive delayed growth observed in the spondyloepiphyseal dysplasia tarda, caused by mutations in TRAPPC2.

The mechanisms underlying the TRAPP-dependent maturation of SGs remain to be identified. Two possible non-exclusive scenarios are worthy of consideration, the engagement of specific protein-protein interactions between TRAPP components and signaling molecules or a central role of TRAPP, originally identified as a tethering complex, in mediating tethering/coalescence of smaller SGs into larger ones, which might be the only ones competent for recruiting signaling molecules.

Supplementary Materials

Materials and methods

Reagents and antibodies

Primary antibodies used in this study were: monoclonal mouse antibody anti-G3BP (BD Transduction Laboratories cat. no. 611126), monoclonal mouse

antibody anti-TRAPPC5 (Abnova cat. no. H00126003-A01), polyclonal goat antibody anti-eIF3 (Santa Cruz cat. no. sc-16377), rabbit polyclonal antibody anti-Sec24C (Sigma-Aldrich cat. no. HPA040196), mouse monoclonal anti-flag (Sigma-Aldrich cat. no. F1804), rabbit polyclonal antibody anti-phosphor-Ser CDK substrates motif (Cell signaling cat. no. 9477), mouse monoclonal anti-GM130 (BD Transduction Laboratories cat. no. 610823), human monoclonal antibody anti-Rab1-GTP (Adipogen cat. no. AG-27B-0006), sheep polyclonal anti-TGN46 (Serotec cat. no. AHP500), rabbit polyclonal antibody anti-Sec31A (Sigma-Aldrich cat. no. HPA005457), mouse monoclonal antibody anti-Retinoblastoma (Cell Signaling cat. no. 9309), rabbit polyclonal antibody anti-phospho Retinoblastoma (Cell Signaling cat. no. 9516), rabbit polyclonal antibody anti-eiF2α (Cell Signaling cat. no. 9722), rabbit polyclonal antibody anti-phospho-eiF2α (ser51) (Cell Signaling cat. no. 3597), mouse monoclonal antibody anti-puromycin (Millipore cat.no. MABE343), rabbit polyclonal anti-□-actin (Sigma-Aldrich cat. no. A2066), mouse monoclonal antibody anti-GAPDH (Santa Cruz cat. no. sc-32233). Synaptopodin. Polyclonal antibodies against TRAPPC2 and TRAPPC3 (Venditti et al., 2012) were obtained in our labs.

Media, serum and reagents for tissue culture were purchased from Thermo Fisher Scientific.

Sodium arsenite was from Sigma-Aldrich (cat. no. S7400), Flavopirodol hydrochloride (cat. no. S2679), SNS-032 (BMS-387032) (cat. no. S1145) and Dinaciclib (SCH727965) (cat. no. S2768) were purchased from Sellekchem. Puromycin was purchased from Sigma-Aldrich (cat.no. A1113802).

Plasmid construction

TRAPPC3-GFP, Sec23A-GFP, Rab1-GFP, OCRL-GFP, Rab5-GFP and Rab7-GFP were obtained as previously described (De Leo et al., 2016; Venditti et al., 2012; Vicinanza et al., 2011).

Cell culture, transfection, and RNA interference

HeLa cells were grown in high glucose (4,500 mg/L) DMEM supplemented with 10% FCS. U2OS were grown in McCoy's supplemented with 20% FCS. Human fibroblasts were grown in DMEM and M199 (1:4) supplemented with 10% FCS.

Podocytes were grown in RPMI-1640 supplemented with 10% Insulin-transferrin-Selenium (ITS) and 10% FCS.

For transfection of DNA plasmids, HeLa cells were transfected using either TransIT-LT1 (Mirus Bio LLC, for BioID2 experiment) or JetPEI (Polyplus, for immunofluorescence analysis) as transfection reagents, and the expression was maintained for 16 hr before processing. Microinjection of TRAPPC3 antibody was performed as described (Venditti et al., 2012).

siRNA sequences used in this study are listed in Fig. SX. HeLa cells were treated for 72 hr with Oligofectamine (Life Technologies) for direct transfection.

Stressing cells

Mammalian cells were treated with 300 uM SA for 30 min at 37°C/5% CO2 (unless otherwise stated). Heat treatment (44 C) was for 45 min.

High content screening

HeLa cells were plated in 384-well culture plates. The day after, a kinase inhibitor library, purchased from Selleckchem, was dispensed using a liquid-handler system (Hamilton) to give a final concentration of 10 µM. 150 minutes after drug administration, cells were treated with sodium arsenite (300 µM) for 30 min at 37°C in the presence of compounds. Finally, samples were fixed for 10 min at room temperature by adding 1 volume of 4% PFA (paraformaldehyde in PBS) to the growth medium and stained with the appropriate antibodies.

For image acquisition, at least 25 images per field were acquired per well of the 384-well plate using confocal automated microscopy (Opera high content system; Perkin-Elmer). A dedicated script was developed to perform the analysis of Sec24C localization on the different images (Harmony and Acapella software; Perkin-Elmer). The script calculated the co-localization value of Sec24C with the SG marker (G3BP). The results were normalized using positive (mock cells exposed to SA) control samples in the same plate.

P-values were calculated on the basis of mean values from three independent wells. The data are represented as a percentage of Sec24C recruitment in the control cells (100%) using Excel (Microsoft) and Prism software (GraphPad software).

Immunofluorescence microscopy

Electron microscopy

Digitonin Assay

HeLa cells were exposed to SA or treated with Dinaciclib (10 μM). After treatment, cells were washed twice with permeabilization buffer (25mM HEPES, 125 mM CH3COOK, 2.5 mM Mg(CH3COO)2, 5 mM EGTA, 1mM DTT) and then permeabilized in PB buffer supplemented with 30 μg/mL of digitonin plus the indicated antibody to visualize permeabilized cells. To analyze Sec24C association with SGs, digitonin was left for 6 min at room temperature. Cells were then washed three times with PB buffer and left in PB buffer for 10 min at RT. Finally, samples were fixed with 4% PFA and processed for IF. To analyse Sec24C association at ERES in the presence of Dinaciclib, cells were fixed for 2 min after digitonin permeabilization.

Puromycin Assay

The puromycin (PMY) assay was modified from Davis (David?) et al., JCB 2012. In brief, mock, TRAPPC2-KD or TRAPPC3-KD HeLa cells were exposed to SA (500 μM, 30 min) in DMEM 10% FCS. Cells were washed three times in DMEM 1X and incubated with 9 μM PMY in DMEM for 5 min at 37°C. Samples were lysed in RIPA buffer and processed for Western blot analysis with the anti-puromycin antibody.

Transport assay

VSVG-mEOS2-2XUVR8 was a gift from Matthew Kennedy (AddGene plasmid # 49803). HeLa cells were transfected with the plasmid for 16 hr and treated with SA for the indicated times. A UV-A lamp was used to illuminate samples (4 pulses, 15 sec each). After the light pulses, cells were left for 10 min at 37°C, then fixed with a volume of 4% PFA and processed for immunofluorescence.

The PC-I transport assay was performed in human fibroblasts as previously described (Venditti et al., 2012). For our purposes, cells were treated with SA

(300μM) for 120 min at 40°C and analyzed 10 min after the temperature switch (40 to 32°C). Cells were then fixed and stained with appropriate antibodies.

Immunoprecipitation

For the studies of TRAPPC2 interactors, HeLa cells were transfected with the TRAPPC2-3XFLAG and 3XFLAG constructs. 16 hr post-transfection, cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% Lauryl Maltoside, protease and phosphatase inhibitors).

Cell extracts from control transfected HeLa cells or cells expressing TRAPPC2-3XFLAG were immune-precipitated for 5 hr at 4°C using M2-flag agarose beads. Immune-precipitates were analysed by Western blot or by LC-MS/MS (Central Proteomics Facility, Sir William Dunn Pathology School, Oxford University).

HeLa cells or podocytes were used to analyse Sec31 phosphorylation status. IP of HeLa cells: cells were plated in 15 mm plates. The day after, samples were treated with Dinaciclib (10 μM in DMSO) for 150 min and with SA (300 μM, 10 min) alone or in combination. Cells were then lysed with RIPA buffer (20mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, protease and phosphatase inhibitors). Cells extracts were immune-precipitated for 30 min at 4°C using the anti-Sec31A antibody. Finally, protein-A Sepharose beads were added to the samples and left for 45 min at 4°C. Samples were analyzed by Western blot using the antibodies indicated in the main text. IP of podocytes: growing and differentiated podocytes were lysed and processed as described for HeLa cells.

Functional annotation of the putative TRAPPC2 protein partners

TRAPPC2-FLAG IP samples were compared with the proteins immunoprecipitated in the control IP (refer to Supplementary Table……): 427 proteins were specifically immunoprecipitated in TRAPPC2-FLAG samples. Moreover, 113 were enriched in the TRAPPC2-FLAG IP samples versus the control IP (ratio >2). Functional Annotation analysis (Dennis et al., 2003; W. Huang et al., 2009) was used to group the putative TRAPPC2 protein partners according to Molecular Function (MF). The DAVID online tool (DAVID Bioinformatics Resources 6.7) was used restricting the output to all MF terms (MF_FAT). Of note,

we found that 251 out of the total 539 proteins were annotated as RNA-binding proteins (GO:0003723) (Ashburner et al., 2000).

Cell cycle analysis by flow cytometry

HeLa cells were harvested and resuspended in PBS. For the fixation, a 9-fold volume of 70% ethanol was added and incubated a 4°C for at least 1hr. Next, cells were centrifuged, washed in PBS and resuspended in PBS containing RNase A 0.1 mg/ml. After incubation for 1hr at 37°C, propidium iodide was added to a final concentration of 10 μg/ml and samples were analyzed in an Accuri C6 flow cytometer.

Cell proliferation analysis by High Content Imaging

Cell cycle analysis by high content imaging was performed using the Click-iT Plus EdU Alexa Fluor 488 Imaging Kit (Life Technologies) according to the manufacturer's instructions. Images were acquired with an automated confocal microscopy (Opera System, Perkin-Elmer) and analyzed through Columbus Image Data Storage and Analysis System (Perkin-Elmer). Nuclear intensity of EdU (5-ethynyl-2'-deoxyuridine, a nucleoside analog of thymidine) in EdU-positive nuclei (S-phase cells) was used as a measure of DNA replication rate.

Yeast Methods

The centromeric plasmid pUG23-Bet3-GFP (His selection) was described previously (Mahfouz et al., 2012). A Pab1-mRFP expressing plasmid was constructed by PCR amplification of the Pab1 gene plus 419 bp of the promoter region from yeast genomic DNA and cloning into the vector backbone of the centromeric plasmid pUG35 (Ura selection), removing the Met25 promoter and replacing GFP with mRFP, using standard cloning techniques. Yeast cells (BY4743) were transformed with both plasmids and grown in –Ura-His media with 2% Dextrose at 30°C to early log phase. Stress granules were induced by incubating cells at 46°C for 10 min (Riback et al., 2017) and observed immediately on an LSM800 microscope.

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Figure legends

Figure 1. The TRAPP complex is recruited to stress granules. (A,B) Localization of endogenous TRAPPC2 under steady state conditions or after sodium arsenite (SA) treatment in (A) a rat chondrosarcoma cell line (300 µM, 1 hr) and (B) in HeLa cells (300 µM, 30 min). Fluorescence microscopy of fixed cells using antibodies against TRAPPC2, eIF3 (to label SGs), Sec31 (to label ERES). DAPI (blue). Small panels in (A) show details and merge of boxed areas. **(C)** Localization of endogenous TRAPPC1 and of GFP-TRAPPC3 to eIF3-labeled SGs in HeLa cells after SA treatment**. (D)** Representative images of a time course analysis of TRAPPC2 redistribution to SGs. Cells were treated as in (B). Graph, quantification of TRAPPC2 localization at SGs over time (the ratio between TRAPPC2 (mean fluorescence intensity) in SG puncta and cytosolic TRAPPC2). Mean ± s.e.m. n = 50 cells per experiment, N = 3. Scale bar, 10 µm in (A-D) **(E)** Extracts from untreated and SA-treated HeLa cells were fractionated by size exclusion chromatography on a Superose 6 column. Fractions were analyzed by Western blot using antibodies against endogenous TRAPPC2, TRAPPC3 and TRAPPC12 (a component of the high MW mammalian TRAPPIII complex, REF), and against the SG marker TIAR-1. TRAPP components co-fractionated with a high molecular weight complex (red dashed box) containing TIAR-1 in SA-treated cells.

Figure 1—figure supplement 1. Re-localization of TRAPP to SGs is independent of stress stimulus and cell type.

Figure 1—figure supplement 2. Stress-induced localization of Bet3 (TRAPPC3) to SGs in yeast cells.

Figure 1—supplement video 1. Bet3 (TRAPPC3) and Pab1 at 30 C in yeast cells.

Figure 1—supplement video 2. Bet3 (TRAPPC3) and Pab1 at 46 C in yeast cells.

Figure 1—figure supplement 3. Association of TRAPP with SGs is reversible after SG disassembly.

Figure 2. TRAPPC2 is required for Sec23/24 relocalization to SGs. (A) HeLa cells, untreated or treated with SA, were fixed and visualized by fluorescence microscopy using anti-Sec24C Ab, anti-eIF3 Ab, and DAPI (blue). **(B)** Quantification of Sec24C redistribution to SGs over time after SA treatment (the ratio between Sec24C (mean fluorescence intensity) in SG puncta and cytosolic Sec24C). Mean ± s.e.m. n = 50 cells per experiment, N = 3. **(C)** Cells treated as in **(A)** stained with an antibody recognizing the coatomer I (COPI), which does not relocalize to SGs (see also Figure 2—figure supplement 1A-N). **(D)** Representative images of TRAPPC2 localization in Sec23AB-KD and Sec24ABCD-KD cells treated with SA. Cells were fixed and visualized by fluorescence microscopy using anti-TRAPPC2 Ab, anti-G3BP Ab (to label SGs) and DAPI (blue). **(E)** Quantification of TRAPPC2 redistribution to SGs after KD of the indicated Sec23 and Sec24 combinations, calculated as the ratio between TRAPPC2 (mean intensity) in SG puncta and cytosolic TRAPPC2. Mean ± s.e.m., n = 40-60 cells per experiment, N = 3. ns: not significant. **(F)** Representative images of Sec24C localization at SGs (stained for G3BP) in TRAPPC3-KD and TRAPPC2-KD cells treated with SA. Depletion of TRAPP or TRAPPC2 reduces Sec24C recruitment. Graph, quantification of Sec24C at SGs, calculated as in (A). Mean ± s.e.m., n = 40-60 cells per experiment, N = 3. **** $P < 0.0001$.

Figure 2—figure supplement 1. Sec24C and Sec23, but not components of other coat complexes or other cytosolic proteins associated with the exocytic and endocytic pathways associate with SGs.

Figure 2—figure supplement 2. Sec16 does not significantly associate with SGs nor is it required for the recruitment of COPII to SGs.

Figure 3. TRAPP and COPII stably associate with SGs. (A) The membrane association of Sec24C was evaluated in non-permeabilized or permeabilized cells with or without SA treatment, as indicated. G3BP was used as an SG marker. Left panel insets, G3BP staining in non-permeabilized cells. Dashed white lines show the outline of permeabilized SA-untreated cells. Blue, DAPI. **(B)** HeLa cells overexpressing GFP-Sar1H79G were treated with SA and immunostained for Sec24C, TRAPPC2, and eIF3, as indicated. Blue, DAPI. Scale bar 10 μm. Graphs show quantification of Sec24C or TRAPPC2 at SGs in GFP-Sar1H79G-expressing cells relative to non-transfected cells. Data are the ratio between Sec24C mean intensity in SG puncta and Sec24C mean intensity at ERES, expressed as a percentage of the non-transfected cells. Mean ± s.e.m., n = 60-70); of Sec24C three independent experiments. ****p<0.0001. mean ± s.e.m. of TRAPPC2 one representative experiment. **(C)** Effect of Sec31A depletion on TRAPPC2 and Sec24C recruitment to SGs. Cells were mock-treated or KD for Sec31, treated with SA (300μM, 30 minutes), and then immunostained for TRAPPC2, Sec24C and eIF3 as indicated. Scale bar, 10 μm. Graph, quantification of Sec24C and TRAPPC2 at SGs, calculated as in (B). Mean ± s.e.m. of TRAPPC2 and Sec24C one representative experiment; n=60-80. ** p<0.001, ****p<0.0001.

Figure 3—figure supplement 1. Effect of Sec31 depletion on Sec24C localization at ERES.

Figure 4. The association of TRAPP with SGs is mediated by its interaction with RNA binding proteins and is under control of CDK1/2. (A) Schematic representation of MS/MS analysis of TRAPPC2 interactors. 251 out of 512 (46%) proteins are RNA-binding proteins, including several SG proteins (yellow ellipse). **(B)** Cells were treated with 273 kinase inhibitors (10 μM) from the Sellekchem library and with SA, and Sec24C localization in G3BP puncta (% of total G3BP area) was calculated (see Materials and methods) and is reported as a percentage of the control (cells treated with SA alone). Gray dashed line, mean of control; gray box, control standard deviation (± 18.2%); dashed red line, threshold of positive hits. **(C)** Enrichment Analysis of the positive hits; for each class of inhibitor, the enrichment (in percentage) of compounds falling into positive hits and the enrichment (in percentage) in the total number of compounds was calculated and expressed as a ratio. **(D)** Evaluation of CDK kinase activity upon SA treatment. Western blot analysis of phosphoSer-CDK substrates in control, SA (300μM, 30 min), Dinaciclib (10 μM, 180 min) and SA+Dinaciclib (150 min Dinaciclib and 30 minutes SA). CDKs are hyperactivated upon oxidative stress and this activation is partially prevented upon CDK inhibition. Left, Western blot, right: Ponceau was used as loading control. Image show one representative experiments out of 3 independent replicates.

Figure 5. (A) All CDK inhibitors present in the library ranked for efficiency in reducing Sec24C recruitment to G3BP spots (% of the control). (A) Drug specificity, from high (++++) to low (+) for the different CDKs as described by Selleckchem and Sec24C recruitment to SGs as a percentage of control. **(B)** Analysis of Sec24C recruitment to SGs in control (MOCK), CDK1-KD, CDK2-KD and CDK1+2-KD HeLa cells. Data are expressed as percentage of Sec24C (mean intensity) in SG puncta normalized for cytosolic signal as a percentage of control conditions. Quantification of one representative experiments. N>100, mean ± s.e.m. ****p<0.0001. **(C)** Representative images of HeLa cells treated with the best three hits (Flavopiridol, SNS-032, Dinaciclib; 1μM 150 min) and then exposed to SA (300μM, 30 minutes), followed by immunostaining for Sec24C and G3BP. Scale bar, 10μm. **(D)** HeLa cells were treated with Flavopiridol, SNS-032, or Dinaciclib for 150 min at the indicated concentrations, subsequently treated with SA (300μM, 30 min), and then immunostained for Sec24C and G3BP and imaged by OPERA. BS-181, a specific CDK7 inhibitor, was used as negative control. Sec24C localization to SGs is expressed as a percentage of the control (cells treated with SA alone). Dashed red line, mean of control; red box, standard deviation of control (± 9.8%). **(E)** Cells were treated as in **(C)** and immunostained for TRAPPC2 and eIF3. Scale bar, 10 μm. The graph shows the quantification of TRAPPC2 localization in SG punta (mean intensity). Data are mean ± s.e.m. expressed as a percentage of TRAPPC2 signal in SGs after Flavopiridol, SNS-032 or Dinaciclib treatment compared to the control (cells treated with SA alone). N=3, three independent experiments, n=60-80 cells per experiments. ****p<0.0001.

Figure 6. TRAPP and Sec23/24 migration to SGs depends on the proliferation state of cells. (A) HeLa cells were starved for 8 h with HBSS and subsequently exposed to SA (30 min, 300µM), and immunostained for Sec24C or TRAPPC2 and eIF3. Scale bar 10 µm. **(B)** Analysis of the proliferation status of the cells after 8 h starvation in HBSS. Left, representative images of starved and non-starved cells using EdU incorporation (see Materials and methods) and, right, quantification of EdU incorporation in starved cells as a percentage of incorporation in control fed cells. **(C)** HeLa cells were seeded at different confluency, treated with SA and stained for Sec24C and G3BP as an SG marker. Scale bar, 10 µm. Flow cytometry (FACS) analysis (right panels) was performed to evaluate the distribution of cell cycle phases in HeLa cell populations seeded at different confluency. The graph shows quantification of Sec24C mean intensity in SG puncta normalized for the cytosolic Sec24C at the indicated cell confluency. Mean ± s.e.m. of a representative experiment out of n=5 biological replicates. n= 50-80. ns, not significant; ****p<0.0001. **(D)** Western blot analysis of phosphorylated retinoblastoma (p-RB) in growing (non-differentiated) versus differentiated podocytes. ß-actin was used as loading control. **(E)** CDK kinase activity in growing and differentiated podocytes. Western blot using a specific antibody recognizing phosphoSer-CDK substrates was used on total cell lysates. **(F)** Growing and differentiated podocytes were treated with SA (300 µM, 30 min) and stained for TRAPPC2 or Sec24C and eIF3. Scale bar, 10µm. The graphs show quantification of TRAPPC2 and Sec24C (mean intensity) in SGs. Mean ± s.e.m. of three independent experiments ****p<0.0001.

Figure 6—figure supplement 1. Differentiation of podocytes.

Figure 7. CDK1/2 modulate the COPII cycle at ERES. (A) Immunofluorescence images of Sec24C and TRAPPC2 in vehicle-treated and SNS-032-treated (1 μM, 3 h) cells. Scale bar, 10 μm. Graphs show the quantification of Sec24C and TRAPPC2 in the peri-Golgi area (mean intensity) normalized in SNS-032-treated cells relative to vehicle-treated cells (set as 100%). Mean ± s.e.m. of three independent experiments. **p<0.05****, p<0.0001. **(B)** HeLa cells were treated with Dinaciclib (1 μM, 3 h) and permeabilized or not with digitonin as described in Materials and methods. A GM130 antibody was added to the buffer of living cells to monitor permeabilization efficiency. Upper panels, non-permeabilized (NP) control cells; middle panels, permeabilized control cells; lower panels, permeabilized Dinaciclib-treated cells. Scale bar, 10 μm. **(C)** Quantification of Sec24C membrane association after CDK inhibitor treatment. Digitonin-permeabilized cells treated with vehicle (CTRL) or the indicated CDK inhibitor (1 μM, 3 h) were immunostained for Sec24C. The mean intensity of Sec24C in the perinuclear area normalized for the cytosolic Sec24C signal in drug-treated cells is expressed as fold change compared to the control; n= 60-80; mean ± s.e.m. of three independent experiments ****p<0.0001. **(D)** Cell lysates from HeLa cells treated with SA (300 μM, 10 min), Dinaciclib (10 μM, 180 min), or their combination were immunoprecipitated using a Sec31 Ab. Western blots of the immunoprecipitates probed using the phosphor-Ser-CDK Ab (upper panel) or the anti-Sec31 Ab (lower panel). Note that the anti-Sec31 Ab immunoprecipitates both Sec31 isoforms. **(E)** Quantification of immunoprecipitated Sec31 from growing and differentiated podocytes using the phosphor-Ser-CDK Ab. Mean ± s.e.m. of three independents experiments. ***p<0.0007.

Figure 8. The recruitment of TRAPP and COPII to SGs slows down ER export
(A) Left, control or SA-treated (500μM, 120 min) human fibroblasts (HFs) were incubated at 40°C for 180 min and stained for eIF3, PCI, and Sec24C. Right, the cells were imaged 10 min after shifting the temperature from 40°C to 32°C and stained for eIF3, PCI, and Giantin (to label the Golgi). Scale bar, 10 μm. **(B)** HeLa cells expressing VSV-G-UVR8 mEOS were left untreated or treated with SA for the indicated times and then pulsed with blue light. Images were taken 10 minutes after the UV pulse and processed for staining. **(C)** Cells were treated with SA for 30 min, the SA was washed out, and cells were left to recover for 180 min in the presence of cycloheximide, followed by a blue light pulse and processing for staining as described in **(B). (D)** Quantification of VSV-G (mean intensity) in the Golgi area to the total VSV-G per cell under the conditions described in (B,C)**.** Data are expressed as percentage of the control. Mean ± s.e.m. of three independent experiment. ****p<0.0001; ns, not significant.

Figure 9. The recruitment of TRAPP induces the fragmentation of the GC in a Rab1-dependent manner. (A) AiryScan images of HeLa cells treated with SA for the indicated times and stained with GM130 (gray). Scale bar, 10 μm. The graph shows quantification of Golgi particles in SA-treated cells. Mean ± s.e.m. of three independent experiments. n=90-100. ****p<0.0001; ns, not significant. **(B)** Control cells and cells treated with Dinaciclib (CDKi) were treated with SA and stained for GM130 (in gray). Insets, eIF3. Blue, nuclear DAPI staining. The graph shows quantification of Golgi particles in HeLa cells untreated or treated with SNS-032, Flavopiridol, or Dinaciclib for 150 min and treated with SA (300 μM, 30 min). Mean ± s.e.m. of three independent experiment. ****p<0.0001. **(C)** Quantification of Golgi particles in cells plated at different percentage of confluency. Mean ± s.e.m. of three independent experiment. ** p<0.001; *** p<0.0002; ns: not significant.
(D) Electron microscopy images of a cell exposed to SA (300 μM, 30 min). GC: Golgi complex. Scale bar 200 nm. **(E)** AiryScan images of HeLa cells at steady state or exposed to SA for the indicated times. A Rab1-GTP specific antibody was used to monitor Rab1-GTP activity and GM130 to stain the Golgi complex. Insets, eIF3. The graph shows quantification of Rab1-GTP at the Golgi complex expressed as a percentage of the control. Mean ± s.e.m. of three independent experiments. *p<0.011, **p<0.002. **(F)** HeLa cells transfected or not with WT GFP-Rab1B were left untreated or treated with SA and stained for GM130 and G3BP to monitor SGs. Dashed white line, WT GFP-Rab1B transfected cells. The graph shows quantification of Golgi particles in the different conditions. NT: non-transfected. Mean ± s.e.m. of three independent experiment. ****p<0.0001; n=30-50.

Figure 9—figure supplement 1. TRAPPC2 migrates to SGs in Rab1 overexpressing cells.

Figure 10. The TRAPP complex controls SG composition and function. (A) Box plot representing SG area (see Materials and methods) in mock, TRAPPC2- and TRAPPC3-depleted cells treated with SA. Distribution of values from three independent experiments. ****p<0.0001. **(B)** HeLa cells microinjected with control preimmune IgG or a TRAPPC3-specific antibody (right panels in green) were treated with SA. The TRAPPC3 Ab disrupts the Golgi (REF), monitored using an anti-TGN46 Ab. Anti-G3BP was used to stain SGs. The graph shows quantification of the SG area. Mean ± s.e.m. three independent experiments; n>80. ****p<0.0001. **(C)** Structured Illumination Microscopy (SIM)-Super resolution (SR) images of endogenous **TRAPPC2 and** GFP-Sec23 localizing at SGs, stained for G3BP. Right, magnification of boxed area. **(D,E)** Localization of Raptor **(D)** and RACK1 **(E)** in mock, TRAPPC3-KD and TRAPPC2-KD HeLa cells treated with SA. G3BP was used to stain SGs. Scale bar, 10 μm. Each graph shows the quantification (mean intensity) of the respective protein in SGs spots expressed as a percentage of the mock. Mean ± S.D. three independent replicates. *p<0.02; **p<0.009 in **(D),** *p<0.05; ****p<0.0001 in **(E). (F,G)** Localization of Raptor **(F)** and RACK1 **(G)** in untreated cells or cells pre-treated with the indicated CDK inhibitor (1 μM, 150 min) and then with SA (300 μM, 30 min). G3BP was used to stain SGs. Scale bar, 10 μm. Graphs show quantification of the localization of the respective protein with SGs, expressed as a percentage of the control. Mean ± s.e.m. of one representative experiment out of three independent replicates, (n=60-80). ****p<0.0001. **(H)** Analysis of cell death after an overnight recovering of HeLa cells treated or untreated with CDKi (SNS-032; Flavopiridol or Dinaciclib, 1 μM, 150 min) and then treated with SA (500 μM, 3 h). Images were automatically acquired by OPERETTA microscopy. Values indicate the percentage of the total number of nuclei (stained with DAPI) positive for BoBo-3 staining. Mean ± s.d. of one representative experiment out of three independent replicates. ns, not significant, ****p<0.0001.

Figure 10—figure supplement 1. TRAPP depletion does not affect protein translation inhibition caused by SA treatment.

Figure 1—figure supplement 1. Re-localization of TRAPP to SGs is independent of stress stimulus and cell type. (**A**) HeLa cells exposed to heat shock (44°C, 45 min), **(B)** human fibroblasts treated with SA (500 μM, 180 min), **(C)** U2OS cells treated with SA (300 μM, 60 min), stained for eIF3 and TRAPPC2.

Figure 1—figure supplement 2. Stress-induced localization of Bet3 (TRAPPC3) to SGs in yeast cells. Cells co-expressing Bet3-GFP and the stress granule marker RFP-PAB1 were exposed to heat shock (46°C, 10 min) and imaged.
Figure 1—supplement video 1.
Figure 1—supplement video 2.

Figure 1—figure supplement 3. Association of TRAPP with SGs is reversible after SG disassembly. Cells were treated with SA for 30 min, the SA was washed out, and cells were left to recover for 120 min and processing for staining. Scale bar 10 μm.

Figure 2—figure supplement 1. Sec24C and Sec23, but not components of other coat complexes or other cytosolic proteins associated with the exocytic and endocytic pathways associate with SGs. (**A-L**) Cells at steady state or treated with SA were visualized by fluorescence microscopy using antibodies against the endogenous proteins or Flag-tagged proteins, or visualizing transfected GFP-tagged proteins, and co-stained for eIF3 or G3BP, as indicated. (**M**) Cells exposed to heat shock (44°C, 45 min) were stained for Sec24C or COPI, and G3BP. Blue, DAPI. (**N**) Schematic diagram showing the cellular location of the tested proteins that associate (red) or not (black) with strss granules.

Figure 2—figure supplement 2. Sec16 does not significantly associate with SGs nor is it required for the recruitment of COPII to SGs. **(A)** Cells were treated with SA (300μM) and co-stained for endogenous Sec16 and G3BP as an SG marker. **(B)** Cells exposed to heat shock were processed as described in (A). **(C)** Representative images of Sec24C localization in Sec16-KD cells treated with SA. Cells were fixed and visualized by fluorescence microscopy using anti-Sec24C Ab, anti-G3BP Ab, and DAPI (blue). Quantification of TRAPPC2 redistribution to SGs after KD calculated as the ratio between Sec24C (mean intensity) in SG puncta and cytosolic Sec24C. Mean ± s.e.m., n = 40-60 cells per experiment, N = 3. ns: not significant

Figure 3—figure supplement 1. Effect of Sec31 depletion on Sec24C localization at ERES. Cells were mock-treated or KD for Sec31 and then immunostained for Sec24 and cTAGE to stain ERES. Scale bar 10μm. The graph shows the mean intensity of Sec24C at ERES normalized for cytosolic signal as a percentage of control conditions.
Figure 6—figure supplement 1. Differentiation of podocytes. Western blot of growing and differentiated podocytes. Synaptopodin was used as a differentiation marker, GAPDH as a loading control.
Figure 9—figure supplement 1. TRAPPC2 migrates to SG in Rab1 overexpressing cells. HeLa cells overexpressing GFP-Rab1B-WT were stressed

with SA (300 μM 30 min) and stained for TRAPPC2 (in red) and eIF3 (in gray). Dashed white line: Overexpressing cells.

Figure 10—figure supplement 1. TRAPP depletion does not affect protein translation inhibition caused by SA treatment. **(A)** Puromycin assay (see Materials and methods) of MOCK, TRAPPC3 and TRAPPC2-KD HeLa cells at steady state and upon SA treatment. **(B)** Evaluation of phosphorylated eIF2a in TRAPPC3 and TRAPPC2 depleted cells exposed to SA. ß-actin was used as a loading control. **(C)** Structured Illumination Microscopy (SIM)-Super resolution (SR) images of endogenous RACK1 and G3BP. Scale bar 500 nm.